

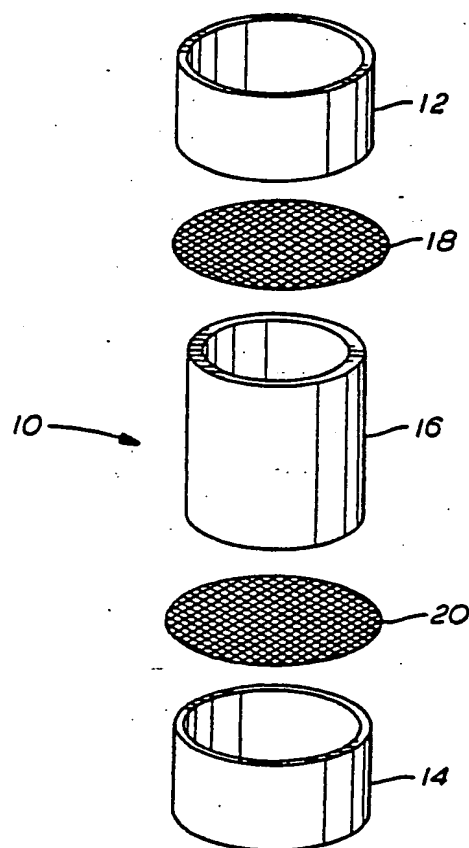
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(54) Title: POROUS WAFER FOR SEGMENTED SYNTHESIS OF BIOPOLYMERS

(57) Abstract

A wafer (10) for synthesizing biopolymers includes a retaining ring (16), solid phase support material (22) and porous members (18, 20) at respective ends of the retaining ring (16). The porous members (18, 20) are maintained in position by rings (12, 14) which circumscribe retaining ring (16). The porous members (18, 20) and the retaining ring (16) cooperate to define therebetween a reaction chamber which houses the solid phase support material (22).



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POROUS WAFER FOR SEGMENTED SYNTHESIS OF BIOPOLYMERS

15

BACKGROUND OF THE INVENTION

The present invention relates to the chemical synthesis of biopolymers, and specifically, to a device for the simultaneous synthesis of large numbers of biopolymers, for example, polynucleotides, polypeptides and polysaccharides.

The development of methods for the chemical synthesis of biopolymers of any desired sequence has resulted in great advances in many areas of biology and medicine during recent years. For example, physical and biochemical studies of the structure and interactions of synthetic DNA fragments has led to important new findings concerning the molecular mechanisms of genetic processes, including DNA metabolism, and regulation of gene expression. Synthetic polynucleotides have played a key role in studies of genetic organization through their use as primers for DNA sequencing and as hybridization probes, linkers and adapters in the cloning of genes. An additional use of synthetic polynucleotides is in DNA probe technology in the diagnosis of disease. Ultimately, synthetic polynucleotides may be used in gene replacement

1 therapy to cure genetic disorders, and in other genome
engineering procedures to provide resistance to disease
and starvation. Synthetic polynucleotides are routinely
used for site-directed in vitro mutagenesis, for studying
5 the structure-function relationships within genetic
regulatory elements and for determining the effects of
specific amino acid substitutions on the functions of
proteins. The latter use, termed protein engineering,
will not only facilitate an understanding of the mechanism
10 of action of enzymes and other proteins, but will also
permit the design of functionally superior proteins and
drugs, leading to important advancements in medicine,
agriculture and industry. Likewise, the availability of
synthetic defined-sequence polypeptides is bringing about
15 equally dramatic advancements in protein chemistry,
immunology, pharmacology and biotechnology.

In many genetic engineering projects it is
necessary to use a large number of different defined
sequence polynucleotides, sometimes hundreds of different
20 sequences in a single experiment. Similarly, some protein
chemistry experiments require hundreds of different
peptide sequences. In order to determine the nucleotide
sequence of the human genome (a project soon to be
initiated, with involvement of many laboratories), on the
25 order of fifty million different polynucleotide primers
will be required. The latter endeavor, along with many
other worthwhile projects that could be carried out by
individual laboratories, are economically impractical with
the current cost to the investigator of synthetic
30 polynucleotides (\$5-\$20 per nucleotide residue).

The capability to chemically synthesize
polynucleotides of defined sequence resulted from the
pioneering work of Michelson and Todd in the 1950s,
(Michelson, A.M. & Todd, Sir A. R., "Nucleotides Part
35 XXXII. Synthesis of a Dithymidine Dinucleotide Containing

1 a 3':5' Internucleotide Linkage," J. Chem. Soc. 1955, pp.
2632-2638), in which a method was developed for specific
chemical synthesis of 5'-3' internucleotide
phosphodiester linkages. This procedure was developed
5 further over the next 20 years, culminating in the total
synthesis of a gene for transfer RNA by Khorana and
Associates, (Khorana, H.G., "Total Synthesis of a Gene,"
Science, Vol. 203, pp. 614-625, (1979). Recently, the
phosphate diester method has been replaced by the
10 phosphate triester method (Letsinger, R.L. and Ogilvie,
K.K., "A Convenient Method for Stepwise Synthesis of
Oligothymidylate Derivatives in Large-Scale Quantities,"
J. Am. Chem. Soc., Vol. 89, pp. 4801-4803, (1967); Narong,
S.A., Brousseau, R., Hsiung, H.M. and Michniewicz, J.J.,
15 "Chemical Synthesis of Deoxyoligonucleotides by the
Modified Triester Method, Meth. Enzymol, Vol. 65, pp.
610-620, (1980)) and the phosphite triester method
(Letsinger, R.L., Finnan, J.L., Heavener, G.A. and
Lunsford, W.B., "Phosphite Coupling Procedure for
20 Generating Internucleotide Links," J. Am. Chem. Soc., Vol.
97, pp. 3278-3279, (1975); Beaucage, S.L. and Caruthers,
M.H., "Deoxynucleotide Phosphoramidites - A New Class of
Key Intermediates For Deoxypolynucleotide Synthesis," Tet.
Lett., Vol. 22, pp. 1859-1862, (1981)), which have the
25 advantage of more rapid synthesis and fewer side
reactions. Both of these methods can be carried out in
solution as originally devised, but have recently been
adapted for solid phase synthesis of polynucleotides
(Matteucci, M.D. and Caruthers, M.H., "Synthesis of
30 Deoxyoligonucleotides on a Polymer Support," J. Am. Chem.
Soc., Vol. 103, pp. 3185-3191, (1981); Sproat, B.S. and
Bannwarth, W., "Improved Synthesis of
Oligodeoxynucleotides On Controlled Pore Glass Using
Phosphotriester Chemistry and a Flow System," Tet. Lett.,
35 Vol. 24, pp. 5771-5774, (1983)). Solid phase synthesis

1 offers the advantage of greater speed of synthesis because
the growing chain is covalently attached to an insoluble
support, permitting reagents to be washed away between
chemical steps and obviating the need to purify the
5 polynucleotide product after each addition of monomer.
Furthermore, solid phase synthesis permits automation of
the process, so that each base addition (via multistep
reaction cycle) can be carried out in
about ten minutes at ambient temperature. The high
10 efficiency of condensation under these conditions
(currently >99%) permits the automated synthesis of
polydeoxynucleotides of chain length greater than 100.

Chemical procedures used for solid phase
synthesis of polypeptides are frequently based on the
15 original protocol of Merrifield, which was successfully
used for synthesis of enzymically active, 124-residue
ribonuclease A (Gutte, B. and Merrifield, R.B., "The
Synthesis of Ribonuclease A," J. Biol. Chem., Vol. 246,
pp. 1922-1941, (1971)). This procedure uses standard
20 polystyrene-divinylbenzene supports, t-butyloxy-carbonyl
(Boc) amino group protection, and DCC-activated
condensation with symmetric anhydride intermediates. The
procedure has been used successfully in automated peptide
synthesizers, as well as in the multiple simultaneous
25 synthesis method of Houghton described below.

Several alternate procedures for peptide
synthesis have been devised. One particularly
advantageous one (Auffret, A.D. and Meade, L.G.,
"Alternative Strategies in Peptide Synthesis," Synthetic
30 Peptides in Biology and Medicine, Alitalo, K., Partanen,
P. and Vaheri, A. (Eds.), Elsevier Science Publishers,
Amsterdam, 1985) utilizes a composite polyamide-Kieselguhr
support (found to be superior for continuous flow
synthesis), fluorenyl-methoxycarbonyl (Fmoc) amino group
35 protection, and 1-hydroxybenzotriazole-activated

1 condensation with pentafluorophenyl ester (PFPE)
intermediates. The high stability of the active ester
intermediates make them more conveniently used for peptide
synthesis than the relatively unstable anhydride
5 intermediates.

Recent developments in polynucleotide synthesis,
including descriptions of the chemical reactions, are
summarized in review articles by John Smith ("Automated
Solid Phase Oligodeoxyribonucleotide Synthesis", American
10 Biotechnology Laboratory, pp. 15-24 (December 1983)) and
Marvin Caruthers ("Gene Synthesis Machines: DNA Chemistry
and Its Uses", Science, Vol. 230, pp. 281-85 (1985)). One
particularly promising recent advancement is the
development of cost-effective procedures for in situ
15 generation of phosphoramidite intermediates from
inexpensive protected nucleosides (Barone, A.D., Tang,
J.-Y. and Caruthers, M.H., "In Situ Activation of
Bis-Dialkylaminophosphines - A New Method for Synthesizing
Deoxyoligonucleotides on Polymer Supports," Nucl. Acids
20 Res., Vol. 12, pp. 4051-4061, (1984); Nielsen, J.,
Taagaard, M., Marigg, J.E., van Boom, J.H. and Dahl, O.,
"Application of 2-cyanoethyl N, N, N', N' -
tetraisopropylphosphorodiamidite for In Situ Preparation
of Deoxyribonucleoside Phosphoramidites and Their Use in
25 Polymer - Supported Synthesis of
Oligodeoxyri-bonucleotides," Nucl. Acids Res., Vol. 14,
pp. 7391-7403, (1986)).

Another advantageous recent development is the
use of amidine groups to protect exocyclic amino groups
30 (e.g., Caruthers, M.H., McBride, L.J., Bracco, L.P. and
Dubendorff, J.W., "Studies on Nucleotide Chemistry 15.
Synthesis of Oligodeoxynucleotides Using Amidine Protected
Nucleosides," Nucleosides and Nucleotides, Vol. 4, pp.
95-105, (1985)). Amidine protecting groups stabilize
35 deoxyadenosine residues against acid-catalyzed

1 depurination, which occurs during the detritylation step of the synthesis cycle, thereby permitting synthesis of longer polynucleotides.

Finally, a procedure for synthesis of RNA
5 polymers on silica supports, involving a modified phosphoramidite approach, has recently been reported (Kierzek, R., Caruthers, M.H., Longfellow, C.E., Swinton, D., Turner, D.H. and Freier, S.M., "Polymer-Supported RNA Synthesis and its Application to Test the Nearest -
10 Neighbor Model for Duplex Stability," Biochemistry, Vol. 25, pp. 7840-7846, (1986)).

Although the above methods permit the synthesis of one or a few polynucleotide sequences at a time, at moderate cost, there is a great need for technological
15 development in this area, to reduce the cost of synthesis and to permit simultaneous synthesis of large numbers of polynucleotide sequences. Progress toward this aim has recently been made in the form of procedures and devices that permit multiple simultaneous synthesis of
20 polynucleotides or polypeptides.

Frank et al. ("A New General Approach for the Simultaneous Chemical Synthesis of Large Numbers of Oligonucleotides: Segmented Solid Supports", Nucleic Acid Research, Vol. 11, No. 13, pp. 4365-77 (1983)) recently
25 used cellulose filters as a solid phase support for polynucleotide synthesis. A protected nucleoside was covalently linked to the hydroxyl groups of the filter paper by 3'-O-succinate linkage, then elongated by the phosphate triester procedure used previously with loose
30 beaded solid phase support materials. In this paper the authors reported synthesis of two octamers, and proposed that by stacking the paper filters into four different reaction columns, designated for addition of A, G, C and T residues to the growing chain and sorting the discs
35 between reaction cycles, a large number of different

1 polynucleotide sequences could be simultaneously
synthesized. The authors demonstrated that the two
octamers synthesized by this procedure (present within the
same reaction column during most cycles) were obtained at
5 reasonable yield, and DNA sequence analysis proved that
the products consisted of the expected nucleoside
sequences and were not contaminated by each other.

The proposed use of the filter paper method for
simultaneous synthesis of many sequences was later
10 implemented by Matthes et al. ("Simultaneous Rapid
Chemical Synthesis of Over One Hundred Polynucleotides on
a Microscale", The EMBO Journal, Vol. 3, No. 4, pp. 801-05
(1984)). These authors used a phosphate triester
procedure similar to that reported by Frank et al., to
15 simultaneously synthesize over one hundred polynucleotide
sequences within a period of two weeks. Several
limitations of the Matthes et al. procedure exist. Due to
low loading capacity of the filter paper disks and their
unfavorable mass transfer properties (resulting in less
20 than optimal access of reagents to the growing chain), the
coupling efficiency at each step is poor compared with
that attained with the standard solid phase synthesis
procedures, and only a very small quantity of desired
polynucleotide is produced, of limited chain length (up to
25 about 20-mer). The product is heavily contaminated by
shorter failure sequences, and must be purified by
time-consuming procedures before use. Nevertheless, this
procedure has the potential of yielding large numbers of
sequences at low cost. This method apparently has been
30 attempted by many laboratories, but apparently only a
very few laboratories have been able to obtain usable
products using the technique.

A very recent report (Bannwarth, W. and Laiza,
P., "A System for the Simultaneous Chemical Synthesis of
35 Different DNA Fragments on Solid Support," DNA, Vol. 5,

1 pp. 413-419, (1986)) describes a mechanical apparatus that
can simultaneously synthesize several different
polynucleotides. The device consists of a series of
stackable rotatable metal disks, each containing, in
5 radially symmetrical position, a single reaction chamber
plus a number of narrow "bypass" holes. The stacked metal
disks can be rotated to produce vertical alignment of all
reaction chambers designated for addition of a given
nucleoside residue to the support-bound DNA chains
10 contained therein, with these chambers being connected by
bypass holes. Thus, by appropriate rotation of the metal
disks following each reaction cycle (created by sequential
flow of reagents and solvents through the system), a
different DNA sequence is synthesized for each of the
15 stacked metal disks. The chief advantage of this device
over the segmented filter paper method is higher coupling
efficiency, enabled by the placement of controlled pore
glass supports within the reaction chambers. DNA chains
of up to 36 residues long were produced utilizing
20 phosphoramidite chemistry. Another advantage of the
design is its potential for automation. The chief
disadvantage is the relatively low number of simultaneous
synthesis (a maximum of 12 DNA fragments were
simultaneously produced).

25 In another approach, utilized for simultaneous
synthesis of different polypeptides, (Houghten, "General
Method for the Rapid Solid-Phase Synthesis of Large
Numbers of Peptides: Specificity of Antigen-Antibody
Interaction of the Level of Individual Amino Acids", Proc.
30 Natl. Acad. Sci. USA, Vol. 82, pp. 5131-35 (August 1985)),
Houghten employed polypropylene mesh bags containing solid
phase support resins used for standard solid phase
synthesis of peptides. By placing a number of these
resin-containing bags into a single stirred reaction
35 chamber, all peptide sequences to which a given amino acid

1 was to be added could undergo the coupling reaction
simultaneously. The authors used this procedure to
simultaneously synthesize 248 different 13-mer peptides
which were obtained in yield comparable to that attained
5 by standard single-peptide solid phase methods. In this
work, each of the 13-mer peptides actually consisted of a
sequence identical to the "control sequence," except for a
single amino acid replacement. Thus, at each amino acid
addition, the vast majority of the resin-containing bags
10 were placed into the same stirred reaction vessel, while
only those resins containing peptides to which a unique
amino acid was to be added at that position in the
sequence were reacted separately from the bulk of the
material. Although the "different" peptide sequences
15 synthesized in Houghten's original work each consisted of
the same sequence, with a single amino acid change from
the "control sequence," it was proposed that by use of a
multiplicity of stirred reaction vessels, each containing
many resin-containing bags, the procedure could be used
20 for simultaneous synthesis of a large number of completely
unique peptide sequences. Houghten's "tea bag" method,
including description of its use for simultaneous
synthesis of 120 entirely different 15-residue peptides,
is further described in a recent article (Houghten et al,
25 "Simultaneous Multiple Peptide Synthesis: The Rapid
Preparation of Large Numbers of Discrete Peptides for
Biological Immunological, and Methodological Studies,"
Biotechniques, Vol. 4, No. 6, pp. 525-28 (1986)).

Two difficulties may prevent the Houghten "tea
30 bag" method from being implemented for simultaneous
synthesis of large numbers of polynucleotide sequences.
The sealable polypropylene mesh bags are not sufficiently
inert to be used in the phosphate triester and phosphite
triester procedures currently used for polynucleotide
35 synthesis. Support-containing porous bags constructed of

1 inert materials such as TEFLON are difficult, if not
impossible to seal, making it difficult to prevent loss of
solid phase support from the bags during synthesis. A
more serious problem is that in the solid phase procedure
5 for polynucleotide synthesis, sufficient space must be
left in the column above the support bed, such that as
solvents and reagents are pumped from below, the support
is "lifted" by the upward flow, resulting in the necessary
mass transfer within the beads required for nearly
10 quantitative chemical reactions. The physical properties
of the non-rigid "tea bags" would not permit the necessary
lifting of support materials during passage of solvents
and reagents through the column.

Accordingly, due to the shortcomings of the
15 present devices and procedures, there exists a need for a
device and procedure for rapid, simultaneous synthesis of
large numbers of any biopolymer of different sequences at
high yields and lower costs.

SUMMARY OF THE INVENTION

20

It is therefore an object of the present
invention to provide an improved device for the chemical
synthesis of biopolymers.

25

Another object of the present invention is to
provide an improved device for the simultaneous synthesis
of large numbers of biopolymers.

Yet another object of the present invention is to
provide for the simultaneous production of large numbers
of defined-sequence biopolymers at very low cost.

30

Another object of the present invention is to
provide a device applicable for the simultaneous, solid
phase synthesis of any of the various biopolymers.

35

Still yet another object of the present invention
is to provide for the simultaneous production of large
numbers of defined-sequence biopolymers at high yields.

1 Yet an additional object of the present invention
is to provide an improved segmented device for
simultaneously producing biopolymers.

5 A further object of the present invention is to
provide an improved device for simultaneously producing
large numbers of biopolymers requiring lower amounts of
reagents and solvents.

10 Yet a further object of the present invention is
to provide an improved device for simultaneously producing
large numbers of biopolymers requiring less synthesis time.

15 A still further object of the present invention
is to provide an improved device for simultaneously
producing large numbers of biopolymers in which the many
segments, hereinafter referred to as "wafers", are easy to
separate from one another after each reaction cycle.

An additional object of the present invention is
to provide an improved solid phase support segment
("wafer") for the chemical synthesis of biopolymers.

20 Thus, in accomplishing the foregoing objects,
there is provided in accordance with one aspect of the
present invention, a segmented wafer synthesis device for
the synthesis of multiple defined-sequence biopolymers,
comprising a solvent delivery system, at least one column
connected to the solvent delivery system to provide
25 solvent and reagent flow through the column, and at least
one wafer positioned in the column at which polymeric
synthesis occurs. In a preferred embodiment, the
synthesis device comprises at least four columns for
receiving four reagents, and a plurality of wafers in each
30 column, wherein each of the wafers provides for the
synthesis of a defined-sequence polymer. The device can
be either automatic, semi-automatic or manual, depending
on user needs.

35 In a further embodiment, the device comprises a
plurality of stacked wafers which are connected together

1 to form a column with the solvent delivery system being
connected to the column to provide flow through the
column.

5 In accordance with another aspect of the present
invention, there is provided a wafer for synthesizing
biopolymers, for example, polynucleotides, polypeptides
and polysaccharides, comprising a solid phase support
material, a retaining ring for retaining the support
10 material in a chamber formed by the inner walls of the
retaining ring, and means, for example, a membrane or
frit, for allowing flow through the retaining ring to the
support material and for preventing migration of the
support material from the retaining ring. Preferably, the
retaining ring comprises an inner, enclosed reaction
15 chamber for receiving and retaining the support material,
the retaining ring being open on both ends. The porous
flow means is an essentially inert porous material, and is
preferably provided at each end of the retaining ring and
extends across the inner chamber to enclose the chamber.
20 In addition, the wafer preferably comprises an inert
securing means for securing the support materials to the
retaining means.

The solid phase support material advantageously
is selected from the group consisting of silica,
25 controlled pore glass (CPG), polystyrene-divinyl-benzene,
polyamide resins, polyamide-Kieselguhr composite resins,
macroreticular resins, benzhydrylamine resins, and
macroporous plastic resins such as MONOBEADS resin (a
resin produced by Pharmacia). The porous support material
30 comprises a derivatized material which includes a
covalently attached residue, for example, a nucleoside in
the case of polynucleotide synthesis.

The porous membrane or frit preferably comprises
flexible membrane composed of TEFLON or other inert
35 fluorocarbons, or rigid frits of glass, stainless steel or

1 titanium. The porosity of the membrane or frit is
sufficiently large to allow flow through the wafer and
sufficiently small to retain the porous support material
in the wafer.

5 In one preferred embodiment, the wafer comprises
a solid phase support material, an inner housing ring
comprising an inner reaction chamber formed by the inner
walls of the ring for receiving and retaining the support
material, the housing ring being open on both ends, an
10 inert porous membrane or frit positioned at and extending
across each of the open ends of the housing ring, the
membrane having a larger diameter than the inner ring, and
two outer rings having an inner diameter slightly larger
than the inner ring, the inner rings encompassing the
15 inner ring and securing the edges of the membrane between
the inner ring and the outer rings. Particularly
preferred is an outer securing means which comprises a
retaining ring positioned about the outer surface of each
end of the housing ring.

20 In another preferred embodiment, the wafer
comprises a solid phase support material, an inert
cylindrical housing ring, open on both ends, and an inert
circular frit snapped into indentations near the open ends
of the housing ring.

25 The wafer design of the present invention
provides for the simultaneous production of numerous
biopolymers. The geometry of the support material results
in high coupling efficiency, and the rigid wafers are easy
to sort after each reaction cycle. This arrangement
30 permits the simultaneous synthesis of many different
sequences. By using support material of varying capacity
(density of derivatization) and by varying the height of
each wafer, the scale of synthesis can be varied from less
than 0.1 micromole to greater than 10 micromoles per
35 segment. Furthermore, segments of varying heights can be

1 stacked within each column, permitting the simultaneous
synthesis of products of widely different scale. The
flexibility and efficiency of this approach should permit
the synthesis of large numbers of biopolymers at a
5 substantially reduced cost. For example, the present cost
of polynucleotide synthesis, under ideal conditions (such
as existence of an in-house synthesis service) is
typically \$10 to \$15 per residue. With the
segmented wafer device, the cost is significantly less,
10 and possibly as low as \$0.50 - \$2.00 per residue. Since
cost presently remains the limiting factor in the use of
synthetic biopolymers, development of the segmented wafer
device is another quantum leap in the use of biopolymers
in scientific research, and should accelerate future
15 developments in biomedical science.

Further objects, features and advantages will
become apparent from a review of the detailed description
of the preferred embodiments which follows, in view of the
drawings, a brief description of which follows.

20 BRIEF DESCRIPTION OF THE DRAWINGS

Referring to the drawings:

25 Figure 1 is an exploded perspective view of an
embodiment of the wafer of the present invention.

Figure 2 is a cross-sectional view of an
embodiment of the wafer of the present invention in its
assembled state.

30 Figure 3 is a perspective view of an embodiment
of the wafer of the present invention in its assembled
state.

Figure 4 is a schematic view of a column assembly
of the segmented wafer synthesis device according to the
present invention.

1 Figure 5 is a schematic view of a segmented wafer
synthesis device according to the present invention.

 Figures 6, 7 and 8 are photographs illustrating
the UV shadowing visualization of DNA produced by the
5 present invention.

 All numerical references in the figures will be
consistent such that the same part in different figures
will have the same number.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

10 The present invention will first be described by
reference to the drawings. At various points in the
following disclosure, the present invention is discussed
in terms of polynucleotide synthesis. The invention, as
has been noted, is equally applicable and useful for the
15 production of any biopolymer that can be synthesized on
solid phase supports. Furthermore, the following
discussion and drawings primarily describe and illustrate
one specific wafer design. It is understood that this
description is for illustrative purposes only, and other
20 wafer designs are possible and within the scope of the
present invention.

 Figure 1 illustrates the wafer 10 of the present
invention prior to assembly, i.e., in an exploded
schematic view. The wafer comprises an outer securing
25 means comprising upper and lower retaining rings 12 and
14. Located between the opposing retaining rings 12 and
14 is an internal housing ring 16, which together with
membranes 18 and 20 serves as the reaction chamber. The
wafer further includes porous materials or membranes 18
30 and 20 positioned at and extending across either end of
the housing ring 16 and secured between the housing ring
16 and the retaining rings 12 and 14.

 The housing ring 16 has an outer diameter just
slightly smaller than the inner diameter of the retaining
35 rings 12 and 14. The porous membranes 18 and 20 have

1 outer diameters greater than the outer diameters of the retaining rings.

It is to be noted that ring, as used here in referring to both the inner housing ring and the retaining rings, includes both circular, rectangular, square and other
5 geometric variations in ring design. The important design criteria is that the rings have a hollow interior space for retaining the reactant components, as described below.

The wafer 10 is shown in its assembled state in
10 Figures 2 and 3. To assemble the wafer, the porous membrane 20 is placed onto the lower retaining ring 14 such that the edges of the membrane extend past the ring around its entire outer circumference. The housing ring 16 is then placed on the lower membrane 20 and pushed into
15 the lower retaining ring 14. The diameters of rings 12, 14 and 16 are selected, with the membrane, to form a fluid tight seal between the rings. After placement of solid phase support material into the housing ring, the upper membrane 18, retaining ring 12 and housing ring 16 are
20 similarly sealed by placing membrane 18 over ring 16 and pushing ring 12 into place. In addition to creation of the fluid tight seal, the design facilitates the retention of the membranes firmly in place during the chemical reaction. This result is achieved by overlapping the
25 edges of the membranes over the housing ring 16 and anchoring the membranes between the retaining rings 12 and 14 and housing ring 16.

As Figures 2 and 3 illustrate, in the assembled wafer 10, the membranes 18 and 20 extend across the ends
30 of the inner housing ring 16, with the ends of the membrane held between the outer retaining rings 12 and 14 and the inner housing ring 16.

The assembled wafer contains the reactant components 22. The reactant components are solid phase
35 supports which have been derivatized by covalently linking

1 a residue, e.g., nucleoside, to the solid support via an
organic spacer arm. The residue, or first base, from
which polymeric growth will begin, is thus separated from
the surface of the support material. The reactant
5 components 22 are placed in the inner housing ring 16
prior to sealing the wafer with membrane 18 and retaining
ring 12. Thus, the housing ring 16 and membranes 18 and
20 together form a reaction chamber for the reactant
components 22.

10 As previously noted, the above disclosure is
directed to one particular wafer design. It is emphasized
that numerous wafer designs are possible and within the
scope of the present invention. For example, the wafer
could include a snap-together or screw-together design.
15 In particular, an alternative embodiment of wafer 10,
could have rigid porous frits, snapped into indentations
in the inside surface of the housing ring near its upper
and lower edge.

The wafer is a rigid, chemically inert chamber so
20 that it will not interfere with or react with the
chemicals used in the synthesis of the biopolymers. The
outer retaining rings and inner housing ring can be
fabricated from a variety of inert materials, for example,
TEFLON and other fluorocarbons, such as KEVLAR and KALREZ.

25 The size of the wafers can vary over a wide
range. For synthesis of milligram quantities of
polynucleotides, the inner diameter of the inner retaining
ring is preferably in the range of about 2-10 mm, and the
height is from about 2-10 mm. For
30 gram quantities of product, the inner diameter is
preferably from about 20-100 mm, and the height is from
about 20-100 mm. Furthermore, the height of the column of
stacked wafers can be increased to permit simultaneous
synthesis of larger numbers of different polynucleotide
35 chains. One skilled in the art will recognize, of course,

1 that the size of the wafers may be smaller or larger than
the above dimensions depending upon the specifics of the
particular synthesis. Furthermore, one skilled in the art
will recognize that the column dimensions will change
5 depending on the biopolymer to be synthesized and the
solid phase support used. In the production of peptides,
the wafer dimensions will generally tend toward the upper
limits of these ranges.

The porous materials which allow for the flow of
10 reagents through the wafers are also formed from a
chemically inert material. For example, suggested inert
materials include TEFLON and other fluorocarbon materials,
such as KEVLAR, fritted or scintered glass, and titanium
and stainless steel frits. Pore size can vary, but is
15 selected so as to allow sufficient flow of the reagents
and washing solutions through the wafer, while retaining
the support material and growing biopolymer chains within
the wafers. A pore size of 50-100 micrometers is
suggested for use with CPG supports, which are typically
20 120-180 micrometers in diameter. The porous material can
assume a variety of designs as long as the necessary flow
and containment are achieved. As illustrated and
described herein, the porous material can be in the form
of a flexible membrane, a rigid fritted structure, etc.

25 The solid phase support on which the biopolymer
chain is formed can be selected from the variety of known
supports. Suggested supports for polynucleotide synthesis
include polystyrene-divinyl-benzene (DVB),
polyamide-Kieselguhr, silica, controlled pore glass (CPG)
30 and plastic resins such as MONOBEADS (a resin produced by
Pharmacia). CPG, silica and MONOBEADS are particularly
preferred as the solid phase support since they are rigid,
i.e., do not swell or contract. Suggested supports for
polypeptide synthesis include polystyrene and vinylbenzene
35 resins, polyamide resins, polyamide-Kieselguhr resins,

1 benzhydrylamine resins, and macroreticular resins.

Support materials of large pore size, for example
200-2000 A, permit good accessibility by the reagents to
the growing chain and efficient washing away of
5 reactants. Also, these supports permit assembly of
relatively long chains, e.g., 50-200 residues, without
steric hindrance between polymers.

The amount of the support material 22 supplied to
the wafer can vary. Factors to be considered in
10 determining the amount of support material added include
the amount of DNA, RNA, polypeptide, polysaccharide or
other biopolymer needed, flowrate and the extent of
derivitization of the solid phase support, e.g.,
micromoles monomeric residue per gram of support.
15 Advantageous results are achieved where the wafers are
from two-thirds to three-fourths full, thus allowing for
mixing and any possible swelling. The use of rigid solid
phase supports of very large pore size, e.g., silica of
3000-4000 A, permits superior mass transfer within the
20 supports, such that wafers can be completely filled with
derivatized supports.

With reference to Figure 4, once assembled the
wafer is placed in a column 24 through which reagents and
washing solvents are passed to create a reaction cycle.
25 The column 24 is designed to receive a number of wafers
10. As illustrated in Figures 4 and 5, delivery system 26
utilizing, for example, argon pressure, passes the
reagents and washing solution through the column 24 and
wafers 10. Preferably, the flow passes upwardly through
30 the column to facilitate the reaction by causing mixing
and distribution of the porous support material within a
given wafer. Typically, the delivery system is connected
to at least four columns in parallel corresponding to the
four bases, cystosine (C), thymine (T), guanine (G) and
35 adenine (A). Additional columns can be provided if

1 modified bases or mixtures of bases are to be utilized in
the synthesis. Any number of wafers can be placed in each
column depending on the number of biopolymer sequences to
be produced. For example, it is possible to place just
5 one wafer in a column. However, this is typically costly
and inefficient and, as pointed out earlier, is one
problem with some of the presently available designs.
Typically, the number of wafers may be in the order of
15-25 per column. However, fewer wafers or more wafers
10 can be utilized. The number of wafers selected must, of
course, allow for sufficient flow through the column. In
this regard, the outer diameters of the wafers should be
selected to provide a snug fit with the inner column
surface to force flow through the wafers themselves, and
15 not along the sides of the column. If additional flowrate
is required, a different solvent delivery system may be
utilized. Also, the number of wafers, of course, will
vary with varying column heights.

The column 24 can be manufactured from any inert
20 material. For example, glass and stainless steel are two
preferred materials. The column typically includes a
plunger 28, which allows for variable numbers and heights
of wafers within the column.

In a further embodiment, the wafers can snap
25 together to form, by themselves, a segmented wafer column,
thereby obviating the need for a separate supporting
column. In this embodiment, the delivery system 26 would
be connected directly to the segmented wafer column.

In the synthesis, as previously noted, a series
30 of columns are set up containing the wafers. Each column
is provided with a reagent for residue addition. For
example, in the synthesis of DNA, one column will be for
the addition of cytosine (C), another for thymine (T),
another for guanine (G), and another for adenine (A).
35 Similarly for RNA synthesis, the thymine can be replaced

1 with the reagent necessary for adding uracil (U). As
previously noted, however, the number of columns used is
not essential to the present invention. A single column
will suffice, but this increases the time required for
5 completion of the biopolymer synthesis. Reagents must be
switched for each synthesis and fewer samples can be
synthesized in one column than in four columns. Thus, use
of multiple columns facilitates the number of reactions to
be carried out and increases the efficiency of the
10 procedure. A further method involves the addition of
dimers, trimers, etc. In this synthesis additional
columns are added. For example, with a dimer one would
use 20 columns, i.e., one column for each dimer which can
be added and one column for each single nucleoside base to
be added.

15 Returning to the DNA method, wafers are
selectively positioned in one of the T, G, C, or A
columns, depending on the first base to be added. After
the appropriate passage of the reagents and chemicals for
the addition of that base to the polynucleotide chain
20 (constituting a reaction cycle), the wafers are removed
from that column, sorted for the next synthesis step,
inserted into the appropriate column and the synthesis
step repeated. This procedure is repeated until the
desired polynucleotide sequences are synthesized. Thus,
25 with the use of different columns for base addition, each
wafer goes through its individual pattern of synthesis.
This procedure allows for the concurrent synthesis of many
different polynucleotides.

30 The present invention can be used for the
production of any biopolymer by solid phase synthesis.
Particularly preferred syntheses include the synthesis of
polynucleotides, polypeptides and polysaccharides by solid
phase methods, provided that these methods employ a
35 flowthrough design, as implemented in the present

1 invention. A particularly preferred solid phase route for
peptide synthesis using the present invention is the
previously mentioned Fmoc pentafluorophenyl ester method,
utilizing polyamide-Kieselguhr supports.

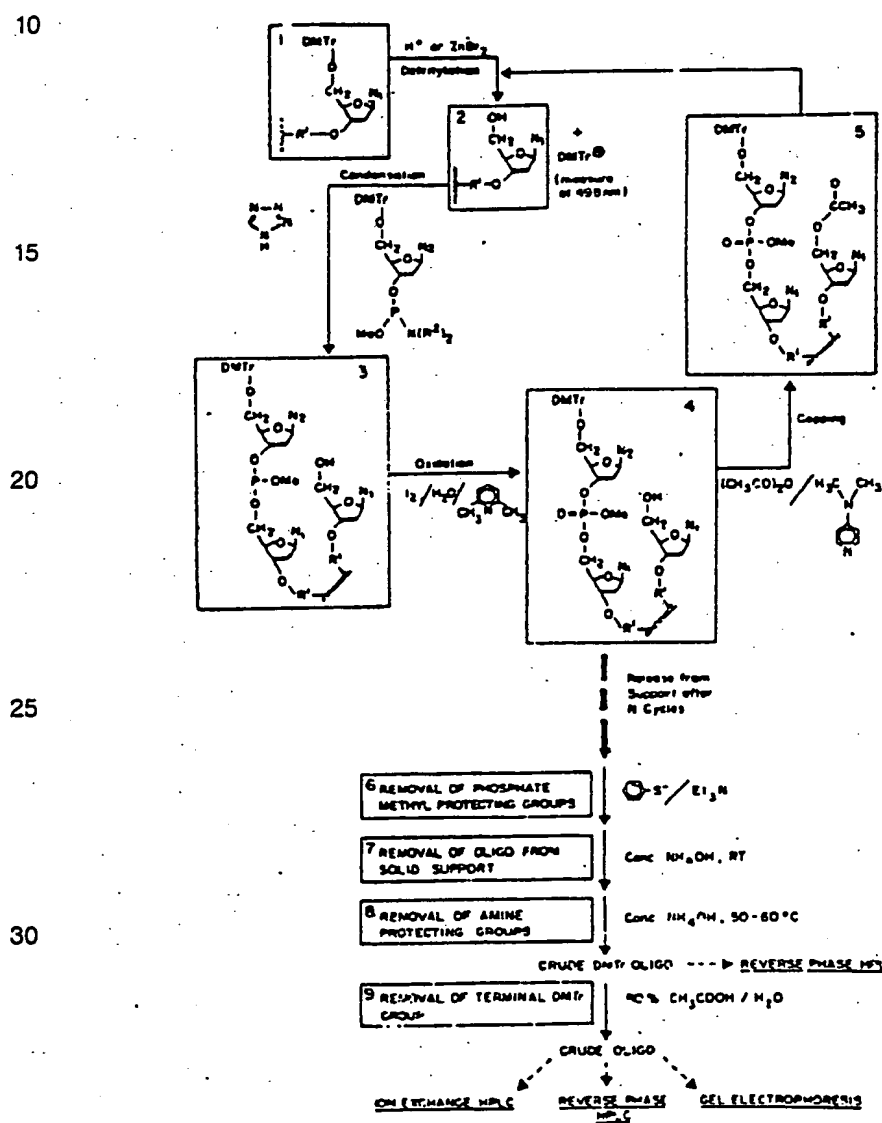
5 The procedure is applicable to the simultaneous
synthesis of multiple defined-sequence biopolymers by
manual, semi-automated or fully automated procedures. For
example, a semi-automated machine can be utilized that is
controlled by a microcomputer. A program editor permits
10 the operator to control the delivery of all reagents to
the solid phase supports. The computer also can provide
the operator, at each step, with instructions for sorting
the wafers and placing them in the correct column. In the
semi-automated system, the operator performs these latter
15 functions. Of course, one skilled in the art recognizes
that the fully automated system is preferred. In this
system sorting of the wafers and their subsequent
placement in the next column is performed by a machine.

The segmented wafer device is designed
20 specifically for biopolymer syntheses that can be achieved
by solid phase, flowthrough methods. The advantage of
solid phase chemistry in the synthesis of biopolymers is
that the step-wise addition to form a biopolymer is
greatly facilitated because the product does not have to
25 be purified after each condensation step. Reactants and
reagents can simply be washed away. This solid phase
synthetic approach has been developed for a number of
different chemistries used in biopolymers synthesis. The
segmented wafer can be used with all these methods.

30 In the synthesis of polynucleotides, the
efficiency of the reaction in each step of solid phase
synthesis has been measured to be between about 95 and
greater than 99%, with the cycle time of approximately
5-30 minutes per nucleoside added. This approach is
35 preferred when the quantity of desired product is in the

1 milligram range, which is ample for most applications. In
 2 addition, solid phase synthesis is highly preferred for
 3 synthesis of mixed probe polynucleotides in which a
 4 mixture of residues exists at certain positions in the
 5 sequence.

The phosphoramidite method of solid phase
 synthesis (diagrammed below) is preferred for use with the
 present invention.



1 The activated intermediate in this approach is a
5'-DMT-2'-deoxynucleoside 3'-phosphoramidite. The method
begins with covalent linkage of the 3'-hydroxyl group of
the first nucleoside to the solid support via a long chain
5 alkyl spacer arm.

 The acid-labile dimethoxytrityl group (DMTr) is
cleaved from the 5'-OH of the support-bound nucleoside by
treatment with dilute aichloroacetic acid. Nucleoside
phosphoramidites (at 10-20 fold molar excess over
10 support-bound nucleoside 5'-OH) are activated by
protonation of their nitrogen atom using tetrazole under
anhydrous conditions, and condensation occurs as shown in
step 2. At the completion of each successive coupling,
the reactive phosphite is converted to a more stable
15 phosphate using a solution of iodine in tetrahydrofuran
and water (step 4). If desired, a "capping" reaction can
next be carried out (with acetic
anhydridedimethylaminopyridine/lutidine, step 5) to
acetylate the 5'-hydroxyl groups that did not react with
20 the activated phosphoramidite in the previous coupling, to
prevent propogation of "truncated" and "nonsense"
(jumbled) sequences.

 At the end of each synthesis cycle, the exocyclic
amino groups of A, C and G remain amide protected, the
25 internucleotide phosphate groups are methyl esterified,
and the 3'-OH end of the growing chain remains
succinate-linked to the support. Prior to addition of the
next residue, the detritylation step is repeated. The
brilliant orange DMTr cation can be quantitated
30 spectrophotometrically to calculate a coupling
efficiency. Using the segmented wafer method, coupling
efficiencies in the range of about 95-99% can be
achieved.

 At the end of the synthesis, the phosphate methyl
35 protecting groups are cleaved by thiophenoxide ion, which

1 forms from thiophenol in the presence of triethylamine
(step 6). This step is not required if 2-cyanoethyl
phosphoramidites are used in the synthesis. Then the
alkali-labile acyl groups (protecting the exocyclic amino
5 groups of A, G and C) and covalent linkage to the solid
support are cleaved by treatment with aqueous ammonium
(steps 7 and 8). If the DMTr group remains, it is cleaved
by concentrated acetic acid (step 9).

As discussed previously, another method (the
10 phosphotriester method) is commonly used for
polynucleotide synthesis. Although the phosphotriester
method could be adapted for use with the present
invention, it is less preferred because of longer cycle
times and greater requirement of anhydrous conditions
15 which are difficult to maintain during sorting of wafers.

The previously mentioned recent developments in
solid phase polynucleotide synthesis, including in situ
phosphoramidite production, amidine protecting groups and
ribopolymer synthesis, could be used in the present
20 invention. Furthermore, it would be obvious to one
skilled in the art to apply the present invention to
future developments in biopolymer synthetic chemistry,
including improvements in condensation, protection and
deprotection reactions or in solid phase supports.

25 Use of the above synthetic methods in the
segmented wafer synthesis of biopolymers can be automated
using commercially available systems. For example, an
automated machine containing four columns, Cruachem model
PS200 synthesizer, can be controlled by an IBM
30 PC-compatible microcomputer. The program editor permits
the operator to control the delivery of all reagents to
the solid phase support. This is required for the
development of the reaction cycle to be used in the
segmented wafer method. Furthermore, the computer can
35 facilitate the sorting process by keeping track of the

1 order of the wafer insertions into the columns. A
computer program is utilized to direct the placement of
each wafer in the appropriate columns during the
synthesis. Thus, a printout indicates which wafers
5 (identified by numbers) are to be placed into a given
column after each reaction cycle, so that wafers are
easily sorted and the separate wafers put in columns for
the appropriate synthetic reaction sequence. The use of
the computer program decreases the amount of error and
10 increases the reliability of the synthesis. Additionally,
development of an automated sorting machine, which is also
controlled by the computer and interfaced with the
existing synthesizer, is possible, to provide for
completely automated synthesis of biopolymers using the
15 segmented wafer device.

The present invention is further described by way
of the following examples.

EXAMPLE I

20 This example describes the standard procedure
used for segmented synthesis of polynucleotides within
chemically inert porous wafers. Details of specific
applications of this procedure are given in subsequent
examples.

25 A. Operation of Interactive Synthesis Setup Program.

DNA sequences to be synthesized are entered
(5' -3' direction) using a word processing program on an
IBM compatible computer. The sequence files are stored in
a non-document file and named in the format. Once the
30 sequences are entered, the Wafer-DNA Setup Program
(written in Basic) is run with the sequence files in the
disk drive. The Wafer-DNA Program examines the sequence
files and generates a hard copy of the following
information: (i) a listing of all sequences entered, along
35 with identifying numbers and names assigned for each, (ii)

1 a listing of numbered wafers to be loaded with each type
of derivatized CPG support (defining the 3'-terminal base
in each sequence), and (iii) a schematic for directing the
sorting of wafers after each reaction cycle.

5 B. Reagent Preparation, Wafer Assembly and
Set-up of Cruachem Model PS200 DNA Synthesizer

Using the software provided with the Cruachem DNA
synthesizer, a method called "Wafer-CE20" has been
created. The method is as follows:

10	Method:	wafer-ce20
	Reservoir 1:	Acetonitrile
	Reservoir 2:	DMAP/THF
	Reservoir 3:	Acetic Anhydride/THF/Lutidine
	Reservoir 4:	Iodine/THF/Lutidine/Water
15	Reservoir 5:	Acetonitrile
	Reservoir 6:	DCA/DCE

Method: wafer-ce20

20 First Cycle

Step 1: Wash Acetonitrile Fixed

Duration = 2:15 Minutes

Step 2: Deblock DCA/DCE Base Variable

25	A	duration = 1:30 Minutes
	G	duration = 1:30 Minutes
	C	duration = 2:30 Minutes
	T	duration = 2:30 Minutes
	Purine (A/G)	duration = 2:30 Minutes
30	Pyrimidine (T/C)	duration = 2:30 Minutes
	N (A/C/G/T)	duration = 2:30 Minutes

Step 3: Wash Acetonitrile Fixed

Duration = 1:30 Minutes

35 Normal Cycle

- 1 Step 1: Reaction Fixed
Duration = 4:00 Minutes
- Step 2: Wash Acetonitrile Fixed
5 Duration = 1:30 Minutes
- Step 3: Wash Acetic Anhydride/THF/Lutidine Fixed
Duration = 0:12 Minutes
- Step 4: Wash DMAP/THF Fixed
Duration = 0:12 Minutes
- 10 Step 5: Wash Acetic Anhydride/THF/Lutidine Fixed
Duration = 0:12 Minutes
- Step 6: Wash DMAP/THF Fixed
Duration = 0:12 Minutes
- Step 7: Wash Acetic Anhydride/THF/Lutidine Fixed
15 Duration = 0:12 Minutes
- Step 8: Wash DMAP/THF Fixed
Duration = 0:12 Minutes
- Step 9: Wash Acetic Anhydride/THF/Lutidine Fixed
Duration = 0:12 Minutes
- 20 Step 10: Wash Acetonitrile Fixed
Duration = 0:12 Minutes
- Step 11: Cap/functionalize Fixed
Duration = 1:30 Minutes
- Step 12: Wash Acetonitrile Fixed
25 Duration = 1:30 Minutes
- Step 13: Wash Iodine/THF/Lutidine/Water Fixed
Duration = 2:00 Minutes
- Step 14: Wash Acetonitrile Fixed
Duration = 1:30 Minutes
- 30 Step 15: Cap/functionalize Fixed
Duration = 60:00 Minutes

1 Step 16: Deblock DCA/DCE Base Variable
A duration = 1:30 Minutes
G duration = 1:30 Minutes
C duration = 2:30 Minutes
5 T duration = 2:30 Minutes
Purine (A/G) duration = 1:30 Minutes
Pyrimidine (T/C) duration = 2:30 Minutes
N (A/C/G/T) duration = 2:30 Minutes
Step 17: Wash Acetonitrile Fixed
10 Duration = 1:30 Minutes

Final Cycle

Step 1: Reaction Fixed
15 Duration = 4:00 Minutes
Step 2: Wash Acetonitrile Fixed
Duration = 1:30 Minutes
Step 3: Wash Iodine/THF/Lutidine/Water Fixed
Duration = 2:00 Minutes
20 Step 4: Wash Acetonitrile Fixed
Duration = 4:00 Minutes

Before synthesis begins it is necessary to assemble the wafers. For each wafer, the bottom portion of the wafer is assembled first so that the derivatized controlled pore glass (CPG) support material can be added through the top. Approximately 18 mg of the appropriate CPG is added, as directed by the printout from the Wafer-DNA Setup Program. Finally the wafer is closed by placing another piece of the porous TEFLON cloth over the reaction chamber and securely fastening this with the outer TEFLON retaining ring. The wafers are loaded into the appropriate columns, as directed in step 2 of the printout from the Wafer-DNA Setup Program.

1 To prepare the synthesizer for operation, the
reservoirs are filled with their respective reagents and
the solvent lines are flushed, using the operating program
supplied with the Cruachem synthesizer. The last reagents
5 to be prepared are the phosphoramidites and the sublimed
tetrazole. Table I describes the reagents used for
polynucleotide synthesis by the segmented wafer method.

NORMAL CYCLE: Wafer - CE20 Method

10 SOLVENTS/REAGENTS PER COLUMN OF 10 WAFERS

1. Acetonitrile - 12.5ml (with solvent
organizer stand, use 11 reservoir)
- 15 2. 6.5% Dimethylaminopyridine in THF (w/v) -
1.2ml
3. Acetic anhydride/THF/Lutidine - 1.6ml
- 20 4. Iodine (0.1M in water/lutidine/THF -
1:10:4) - 4ml
5. 3% Dichloroacetic acid/dichloroethane
(w/v) - 4ml

25

SOLVENT FLOW RATE: 2ml/min

AVERAGE CYCLE TIME: 18 min

30

SYNTHESIS SCALE: 0.5-1 micromole per wafer

SUPPORT: Nucleoside-CPG, typically 15-20 mg
per wafer

35

MONOMER SOLUTION: 0.1M CE phosphoramidite

1 6.67 ml acetonitrile/.5 g T-phosphoramidite
 6.00 ml acetonitrile/0.5 g G-phosphoramidite
 5.80 ml acetonitrile/0.5 g A-phosphoramidite
 6.00 ml acetonitrile/0.5 g C-phosphoramidite

5 Catalyst - 0.5M tetrazole (20 ml acetonitrile/0.7 g tetrazole)

10 Mix 0.5ml monomer and 0.5ml catalyst and inject
 into the column.

C. Synthesis of Polynucleotides

 Using the PS200 Cruachem DNA Synthesizer and
resident operating software, the Wafer-CE20 method and the
15 segmented wafer synthesis device depicted in Figure 5, the
segmented synthesis of polynucleotides is carried out,
employing the previously described 2-cyanoethyl
phosphoramidite chemistry. After the wafer-containing
columns are connected to the synthesizer, the first cycle,
20 consisting only of detritylation and washing, is carried
out as indicted in the method ("First Cycle," Steps 1
through 3). Initiation of each subsequent cycle occurs
upon injection of phosphoramidites, immediately preceding
Step 1 ("Normal Cycle"). Step 15 ("Normal Cycle") is not
25 a repeat of capping Step 11, but rather is a variable
"pause" period during which the wafers are sorted, as
directed by the printout from the Wafer-DNA Setup
Program. In each normal cycle, as soon as sorting of
wafers is completed and columns are reconnected to the
30 synthesizer, the synthesis cycle is resumed and
detritylation and washing are carried out. The final
cycle is identical to the normal cycle, except that
capping and detritylation are omitted. If desired, after
synthesis within all wafers is complete, the wafers can be

1 reassembled into columns and subjected to detritylation to remove the remaining 5'-DMT protecting groups.

After the synthesis has been completed, the wafer contents are emptied into screw-top vials and the DNA is
5 cleaved from the support, further deblocked and purified by prior-art procedures, following the instructions provided in the Cruachem PS200 operation manual.

The above procedure has been carried out numerous times, resulting in the simultaneous synthesis (at a scale
10 of 0.5-1.0 micromole) of between 3 and 79 different DNA sequences in a single day, of length ranging from 15 to 25 residues. The coupling efficiency at each step was typically about 95% and DNA sequences have been confirmed by the Maxam-Gilbert sequencing method.

15

EXAMPLE II

Simultaneous Synthesis of Three Test Polynucleotides

To assess the usefulness of the segmented wafer synthesis device for biopolymer synthesis, simultaneous
synthesis of three pentadecamers was carried out, using
20 the equipment illustrated in Figures 1-5 and the general procedure described in Example 1, as further detailed below. The nucleotide sequences of the test DNA molecules were:

1. 5'-GAGCCATCAAGCCAG-3'
- 25 2. 5'-GCTGCAGAGAGGCCA-3'
3. 5'-GAGGTGTTGAGCTG-3'

The details of the synthesis are:

SOURCE OF REAGENTS: Cruachem

30 SCALE OF SYNTHESIS AND WAFER DIMENSIONS: Each wafer (10 mm o.d. x 4 mm h.) contained 18 mg of nucleoside-CPG (approximately 0.6 micromole) and was assembled from components of the following dimensions (see Figures 1-3): Porous Teflon cloth, 12 mm diameter; Inner

35

1 housing ring, 4 mm i.d. x 4 mm h.; Outer retaining rings,
10 mm o.d. x 2 mm h.; Internal volume, .050 ml.

REACTION CYCLE AND REAGENT/SOLVENT USAGE: The
standard "CE Phosphoramidite" protocol and reaction cycle,
5 as specified in the Cruachem PS200 Synthesizer instruction
manual for prior-art operation, was used in this
experiment. The "standing" steps of the reaction cycle
(condensation step 1 and capping step 11) were carried out
for the same times given in Example I for the "wafer-CE20"
10 method (4.0 and 1.5 minutes, respectively). Step 1 was
initiated by mixing 0.1 ml of 0.1M CE phosphoramidite and
0.1 ml of 0.5M tetrazole (both in anhydrous acetonitrile)
in a syringe and injecting the mixture into each column.
The remaining "flow" steps in the reaction cycle were
15 carried out (at 2 ml/min) for one-half the time specified
in Example I for the "normal cycle" of the "wafer-CE20"
method. The columns were briefly flushed with Argon just
prior to the sorting step 15. The average cycle time was
11 minutes. The quantity of reagents consumed per cycle
20 per wafer, along with approximate cost per base addition
(based on catalog price of nucleoside-CPG, reagents and
solvents) were as follows:

3.7 ml acetonitrile
0.4 ml 6.5% dimethylaminopyridine in THF
25 0.5 ml acetic anhydride/THF/Lutidine
1.2 ml iodine (0.1M in water/lutidine/THF-1:10:4)
1.2 ml 6.3% dichloroacetic acid/dichloroethane
0.06 ml 0.5M tetrazole in acetonitrile
0.06 ml 0.1M 2-cyanoethyl phosphoramidite in
30 acetonitrile

Cost per base addition: \$1.98, compared with
\$5.42/base if synthesis were carried out by the Cruachem
PS200 Synthesizer, operated in the standard (prior-art)
35 mode.

1 POST-SYNTHESIS IS DEPROTECTION, DNA PURIFICATION,
ANALYSIS: The final detritylation step was carried out on
the column (as in step 16 of the "normal cycle"). After
wafer contents were emptied into 1.5 ml eppendorf tubes, 1
5 ml of fresh concentrated ammonium hydroxide was added,
tubes were capped and mixed. After 20 minutes at room
temperature (during which cleavage of polynucleotides from
the CPG occurred), the liquid, along with 1 ml additional
concentrated ammonium hydroxide, was transferred to a
10 screw-top glass vial (15 ml o.d. x 45 mm height), tightly
sealed with a Teflon-lined cap, and incubated at 55 degrees
C for 6-15 hours (to deprotect exocyclic amino groups of
C, A and G). The ammonia was removed by vacuum, using a
Savant SpeedVac concentrator (1 hr by water jet, followed
15 by overnight at high vacuum). The dried DNA was dissolved
in a small volume of water, then purified by
electrophoresis (20% polyacrylamide, 7M urea), visualized
by "UV-shadow" gels produced from 20 A₂₆₀ units of the
crude reaction products are represented by Figure 6.

20 The uppermost band in each gel represents the
desired full-length product, the faint lower bands
represent "failure" sequences, and the dark band at the
bottom represents the bromophenol blue marker dye. These
gels were comparable to those obtained with similar DNA
25 products produced on an automated Applied Biosystems Model
380A Synthesizer (using prior-art phosphoramidite
procedure) and during which coupling efficiencies were
measured (by the standard trityl release assay) to be
98-99%. Thus, the average coupling efficiency in the
30 synthesis of the three pentadecamers by the segmented
wafer synthesis device was estimated to be about 98-99%.

35 COMMENTS: These DNA products were successfully
5'-phosphorylated (using T4 polynucleotide kinase) and
used as hybridization probes, by prior-art procedures.
The high yield and quality and reduced cost of the

1 products demonstrates the usefulness of the present
invention for simultaneous polynucleotide synthesis.
Furthermore, an important finding is that the manual
sorting process carried out after each reaction cycle does
5 not negatively affect the synthesis.

EXAMPLE III

Simultaneous Synthesis of 62 Biopolymer

To assess the utility of the present invention
10 for simultaneous synthesis of large numbers of
biopolymers, 62 different DNA nonadecamers were
synthesized, using the equipment illustrated in Figures
1-5 and the general procedure outlined in Example I, as
further detailed below. The nucleotide sequences of the
15 test DNA molecules were:

1. 5'-GAAAGGTTAGATTCCTCAC-3'
2. 5'-AAGAAAGGTCAAATTCCTC-3'
3. 5'-TGGTGGGAAGCAAGGTTAAA-3'
4. 5'-GCTTGGTGGCAGAAAGGTT-3'
5. 5'-GAATGGTTTCTAACTGCTT-3'
- 20 6. 5'-TTTTCAAAGCGAATGGTTT-3'
7. 5'-GGTTTAATGTCCTGTTTTT-3'
8. 5'-GGCGTTTTTCATCAGCGGTT-3'
9. 5'-CCACCCGGCCTTTTCTTCA-3'
- 25 10. 5'-GACGGCGCGTCACCCGGCC-3'
11. 5'-GTTGACGGCTCGCCACCCG-3'
12. 5'-TCTTCCAGTTCCTCTTCCG-3'
13. 5'-AGTTCTTCCCGTACCTCTT-3'
14. 5'-CCAGACCACCATACTCCAG-3'
- 30 15. 5'-GATTTTCAGCATCGCCAGAC-3'
16. 5'-AGCGGCAGCATGTCGGTGT-3'
17. 5'-TGCACACGCTCGGTTTTTCG-3'
18. 5'-TATGCACACCCCCGGTTTT-3'
19. 5'-AAGAGGTATCCACACGCCC-3'
- 35 20. 5'-GGTGATAAGCGGTATGCAC-3'

- 1 21. 5'-CAACGTCCCCTTGCAGTTA-3'
22. 5'-ATAAACGTCTCGTTGCAGT-3'
23. 5'-ACGATAAACCTCCCGTTGC-3'
24. 5'-TTTGCAGGTCAGGATCGGT-3'
- 5 25. 5'-ATCTTTTGCCGTTAGGAT-3'
26. 5'-CGCACCGGACTGTTTTGCA-3'
27. 5'-TCGTTACGCTCCGGAATGT-3'
28. 5'-CTTCGTTACCCACCGGAAT-3'
29. 5'-TACGACGACGTTCTTCGTT-3'
- 10 30. 5'-ACGCCTGGCCGATACGACG-3'
31. 5'-GCAATAAACTCCTGGCGGA-3'
32. 5'-GGCGCAATAGACGCCTGGC-3'
33. 5'-TCCTCTGGCTCAATAAACG-3'
34. 5'-CAATCTGCGCGTAGtCCGC-3'
- 15 35. 5'-ATAATGCGCCGTTCAATCT-3'
36. 5'-CCATAATGCCCAGTTCAAT-3'
37. 5'-GAAAGATGCTCCATAATGC-3'
38. 5'-GAAAGATGCTCCATAATGC-3'
39. 5'-GCGAAAGATCCGCCATAAT-3'
- 20 40. 5'-CGCTACGGCCTTGCTCGCT-3'
41. 5'-ATCGCTTTCTCGCTACGGC-3'
42. 5'-TGATCGCTTCCGCGCTACG-3'
43. 5'-AAATCAGACGAAAGTTGAT-3'
44. 5'-TCATGCCATCAATCAGACC-3'
- 25 45. 5'-CACTCATGCGATAAATCAG-3'
46. 5'-GAGCACGGGCGCGTTCCAT-3'
47. 5'-TTCGCCTGATCACGGGTGC-3'
48. 5'-TGCTCTTTCTCCTGAGCAC-3'
49. 5'-CGTCCGTCCCGCGTTTCAA-3'
- 30 50. 5'-GACGGCGTCTGTCCAGCGT-3'
51. 5'-ACAGACGGCCTCCGTCCAG-3'
52. 5'-GATACAGACCGCGTCCGTC-3'
53. 5'-TTAATGGCTTCACGTTTCA-3'
54. 5'-GCGTTAATGTCTGCACGTT-3'
- 35 55. 5'-TTGGCGCGTCAATGGCTGC-3'

- 1 56. 5'-CGGTTCCCTCCATTGGCGC-3'
2 57. 5'-ATGTCGGCGTCGGTTCCT-3'
3 58. 5'-CGTTTGATACTGTCGGCGG-3'
4 59. 5'-TCGCCCCGTTTCGATAATGTC-3'
5 60. 5'-TCAACGGCACTCATCGCCC-3'
6 61. 5'-TCATCGTGTTTCCTGCATGA-3'
7 62. 5'-GTTTCATCGTCTACCTGCAT-3'

The details of the synthesis are:

10 SOURCE OF REAGENTS: Cruachem

SCALE OF SYNTHESIS AND WAFER DIMENSIONS: As in
Example II, 18 mg of derivatized CPG (approximately 0.6
micromole) was placed into each wafer of dimensions, 10 mm
o.d. x 4 mm height.

15 REACTION CYCLE: The synthesis was carried out
using the "wafer-ce20" method (reaction cycle as listed in
Example 1). A mixture of 0.5 ml 0.5M tetrazole and 0.5ml
0.1M 2-cyanoethyl phosphoramidite was injected upwards
through the column of wafers to initiate step 1. Duration
20 of average reaction cycle was 18 minutes. Average time
required for sorting (step 15) was 12 minutes.

REAGENT AND SOLVENT USAGE PER REACTION CYCLE:
The quantities of reagents and solvents required per base
addition per wafer, and cost of synthesis per base
25 addition were:

0.80 ml acetonitrile
0.08 ml 6.5% dimethylaminopyridine in THF
0.10 ml acetic anhydride/THF/Lutidine
0.26 ml iodine (0.1M in water/lutidine/THF-1:10:4)
30 0.26 ml 6.3% dichloroacetic acid/dichloroethane
0.03 ml 0.5M tetrazole in acetonitrile
0.03 ml 0.1M 2-cyanoethyl phosphoramidite in
acetonitrile

1 Cost per base addition: \$0.65. This value is
only about 1/8 the cost of synthesis that would pertain to
synthesis of these same polynucleotides by the Cruachem
PS200 Synthesizer, or by the fully automated Applied
5 Biosystems Model 380A, operated in the standard
(prior-art) mode.

TOTAL TIME REQUIRED FOR SYNTHESIS OF 62
POLYNUCLEOTIDES: Synthesis was completed in a single day,
over a period of 12 hours. This compares with
10 approximately ten days required to produce this number of
polynucleotides using a 3-column, fully automated Applied
Biosystems Model 380A Synthesizer, operating at two
syntheses per column per day.

POST-SYNTHESIS DEPROTECTION, DNA PURIFICATION,
15 ANALYSIS: Procedures were the same as those given in
Example II. The "UV shadowing" gels illustrated in
Figures 7 and 8, are representative of those obtained with
20 A₂₆₀ units of crude reaction products formed in this
experiment.

20 Based on the results of UV shadowing gel analyses
and quantitation of purified DNA products, the average
coupling efficiency during this multiple simultaneous
synthesis was estimated to be 92-98%.

COMMENTS: The purified polynucleotides were used
25 in prior-art procedures for oligonucleotide-directed
mutagenesis. During this work the DNA products were
successfully 5'-phosphorylated (using T4 polynucleotide
kinase), annealed to the DNA templates, and elongated by
DNA polymerase. Thus, DNA products of high quality were
30 produced, at high yields and at greatly reduced cost and
requiring greatly reduced time, compared to prior-art
procedures.

Thus, the wafers of the present invention provide
for the synthesis of multiple defined-sequenced
35 biopolymers. The geometry of the support material results

1 in high coupling efficiencies, and the rigid wafers
facilitate sorting after each reaction cycle. Extremely
advantageous are the reduced synthesis cost realized by
the present invention and the decreased time required for
5 synthesis of large numbers of biopolymers. The economic
and time-saving advantages created by the segmented wafer
method should increase demand for the commercial product
and fuel future developments in biomedical science.

The present invention, therefore, is well adapted
10 to carry out the objects and attain the ends and the
advantages mentioned as well as those inherent therein.
While presently preferred embodiments of the invention
have been given for the purpose of disclosure, numerous
changes in the details of construction and arrangement of
15 parts can be made which will readily suggest themselves to
those skilled in the art and which are encompassed within
the spirit of the invention and the scope of the appended
claims.

20 What is claimed is:

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1 1. A chemically inert wafer for synthesizing
biopolymers, comprising:

 a solid phase support material;
 a retaining ring for retaining said support
5 material in a chamber formed by the inner walls of
said retaining ring; and

 porous means for allowing flow through said
retaining ring to said support material and for
preventing migration of said support material from
10 said retaining ring.

 2. A wafer as claimed in claim 1, wherein said
retaining ring comprises an inner, enclosed reaction
chamber for receiving and retaining said support material,
15 said retaining ring being open on both ends.

 3. A wafer as claimed in claim 2, wherein said
porous flow means comprises separate means provided at
each end of said retaining ring and extending across said
20 open ends to enclose said chamber.

 4. A wafer as claimed in claim 1, further
comprising securing means for securing said porous flow
means to said retaining ring.
25

 5. A wafer as claimed in claim 4, wherein said
securing means is chemically inert.

 6. A wafer as claimed in claim 1, wherein said
30 retaining ring is chemically inert.

 7. A wafer as claimed in claim 1, wherein said
solid phase support material is selected from the group
consisting of silica, controlled pore glass,
35 polystyrene-divinyl-benzene, polyamide-Kieselguhr,

1 benzyl-linked polystyrene resins, spacer-linked styrene
resins, polyamide resins, and macroreticular resins.

5 8. A wafer as claimed in claim 7, wherein said
solid phase support material comprises controlled pore
glass.

10 9. A wafer as claimed in claim 1, wherein said
solid phase support material comprises a derivatized
material includes a covalently attached residue.

15 10. A wafer as claimed in claim 1, wherein said
porous flow means comprises a fluorocarbon material,
fritted or scintered glass, titanium and stainless steel
frits.

20 11. A wafer as claimed in claim 1, wherein the
porosity of said flow means is sufficiently large to allow
flow through the wafer and sufficiently small to retain
said solid phase support material in the wafer.

12. A chemically inert wafer for synthesizing
biopolymers, comprising:

25 a solid phase support material;
an inner housing ring comprising an inner
reaction chamber formed by the inner walls of said
ring for receiving and retaining said
support material, said retaining ring being open on
both ends;

30 an inert porous membrane positioned at and
extending across each of said open ends of said inner
retaining ring, said membrane having a larger diameter
than said inner ring; and

35 two outer rings having an inner diameter slightly
larger than said inner ring and securing the edges of

1 said membrane between said inner ring and said outer
rings.

5 13. A wafer as claimed in claim 3, wherein said
porous flow means are pressure fitted in said retaining
ring.

10 14. A segmented wafer synthesis device for the
synthesis of multiple defined-sequence biopolymers,
comprising:

 a solvent delivery system;

 at least one column connected to said solvent
delivery system to provide solvent and reagent flow
through said column; and

15 at least one wafer as claimed in claim 1
positioned in said column at which polymeric synthesis
occurs.

20 15. A synthesis device as claimed in claim 14,
further comprising at least four columns for receiving
four reagents, and a plurality of wafers in each column,
wherein each of said wafers provides for the synthesis of
a definedsequence biopolymer.

25 16. A segmented wafer synthesis device for the
synthesis of multiple defined-sequence biopolymers,
comprising:

 a solvent delivery system; and

30 a plurality of stacked wafers as claimed in
claim 1, each of said wafers including a biopolymeric
synthesis material and being connected to the next
adjacent wafer to form a column, wherein said solvent
delivery system is connected to said column to provide
flow through said column.

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AMENDED CLAIMS

[received by the International Bureau on 13 June 1988 (13.06.88);
original claims 1, 12 and 16 amended; other claims unchanged (4 pages)]

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What is claimed is:

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1. (Twice Amended) A modular chemically inert wafer for synthesizing biopolymers adapted to be abutably stacked in a multiple relationship wherein each said wafer has independent upper and lower porous means when in a stacked relationship, comprising:

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a solid phase support material capable of binding a biopolymer residue;

a retaining ring having inner and outer walls, for retaining said support material in a chamber formed by the inner walls and coaxially extending therethrough [of] said retaining ring; and

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upper and lower porous means [for] positioned at each end of said retaining ring wherein said porous means [allowing] allow flow through said retaining ring to said support material and [for] [preventing] prevent migration of said support material from said retaining ring.

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2. A wafer as claimed in claim 1, wherein said retaining ring comprises an inner, enclosed reaction chamber for receiving and retaining said support material, said retaining ring being open on both ends.

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3. A wafer as claimed in claim 2, wherein said porous flow means comprises separate means provided at each end of said retaining ring and extending across said open ends to enclose said chamber.

4. A wafer as claimed in claim 1, further comprising securing means for securing said porous flow means to said retaining ring.

5. A wafer as claimed in claim 4, wherein said securing means is chemically inert.

6. A wafer as claimed in claim 1, wherein said retaining ring is chemically inert.

7. A wafer as claimed in claim 1, wherein said solid phase support material is selected from the group consisting of silica, controlled pore glass, polystyrene-divinyl-benzene, polyamide-Kieselguhr, benzyl-linked polystyrene resins, spacer-linked styrene resins, polyamide resins, and macroreticular resins.

8. A wafer as claimed in claim 7, wherein said solid phase support material comprises controlled pore glass.

9. A wafer as claimed in claim 1, wherein said solid phase support material comprises a derivatized material includes a covalently attached residue.

10. A wafer as claimed in claim 1, wherein said porous flow means comprises a fluorocarbon material, fritted or scintered glass, titanium and stainless steel frits.

11. A wafer as claimed in claim 1, wherein the porosity of said flow means is sufficiently large to allow flow through the wafer and sufficiently small to retain said solid phase support material in the wafer.

12. (Twice Amended) A modular chemically inert wafer for synthesizing biopolymers adapted to be abutably stacked in a multiple relationship wherein each said wafer has independent upper and lower porous means when in a stacked relationship, comprising:

1
a solid phase support material capable of binding a biopolymer residue;

5 an inner housing ring having inner and outer walls comprising an inner reaction chamber formed by the inner walls of said ring and coaxially extending therethrough for receiving and retaining said support material, said retaining ring being open [on] at both ends;

10 an inert porous membrane positioned at and extending across each of said open ends of said inner retaining ring, said membrane having a diameter larger [larger diameter] than and outermost diameter of said [inner] housing ring so that said membrane extends beyond said outer wall; and

15 two outer sleeve-like rings having an inner diameter slightly larger than said inner ring and the thickness of said membrane for securing the edges of said membrane between said inner housing ring and said outer sleeve-like rings.

20 13. A wafer as claimed in claim 3, wherein said porous flow means are pressure fitted in said retaining ring.

25 14. A segmented wafer synthesis device for the synthesis of multiple defined-sequence biopolymers, comprising:

30 a solvent delivery system;
at least one column connected to said solvent delivery system to provide solvent and reagent flow through said column; and

35 at least one wafer as claimed in claim 1 positioned in said column at which polymeric synthesis occurs.

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15. A synthesis device as claimed in claim 14,
further comprising at least four columns for receiving
four reagents, and a plurality of wafers in each column,
5 wherein each of said wafers provides for the synthesis of
a defined- sequence biopolymer.

16. (Amended) A segmented wafer synthesis
device for the synthesis of multiple defined-sequence
biopolymers, comprising:
10 a solvent delivery system; and
a plurality of stacked wafers as claimed in
claim 1, each of said wafers including a
biopolymeric synthesis material and being
connected to the next adjacent wafer to form a
15 column, wherein each said wafer directly abuts
the adjacent wafer and wherein
said solvent delivery system is connected to said
column to provide flow through said column.

Please add the following new claim:

20 17. The wafer as claimed in claim 1 wherein the
porous means comprises said solid phase supporting
material.

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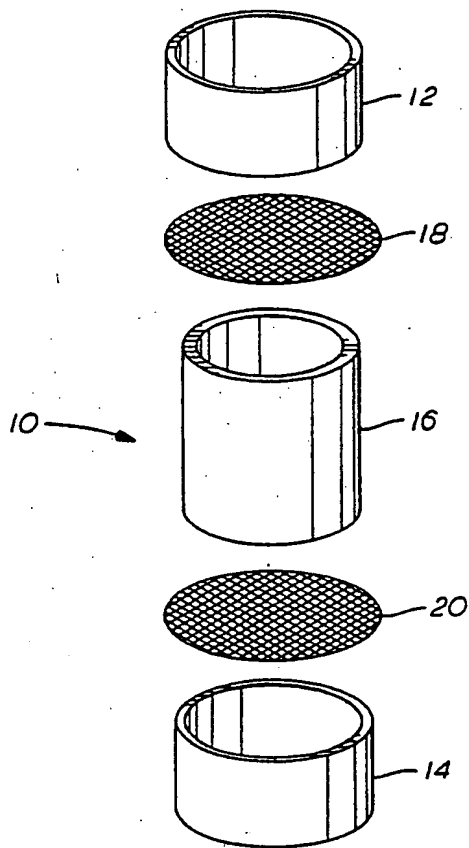


FIG. 1

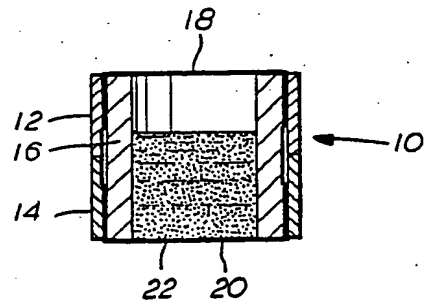


FIG. 2

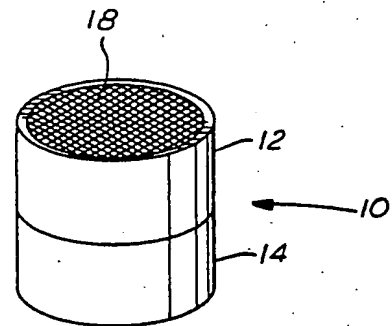


FIG. 3

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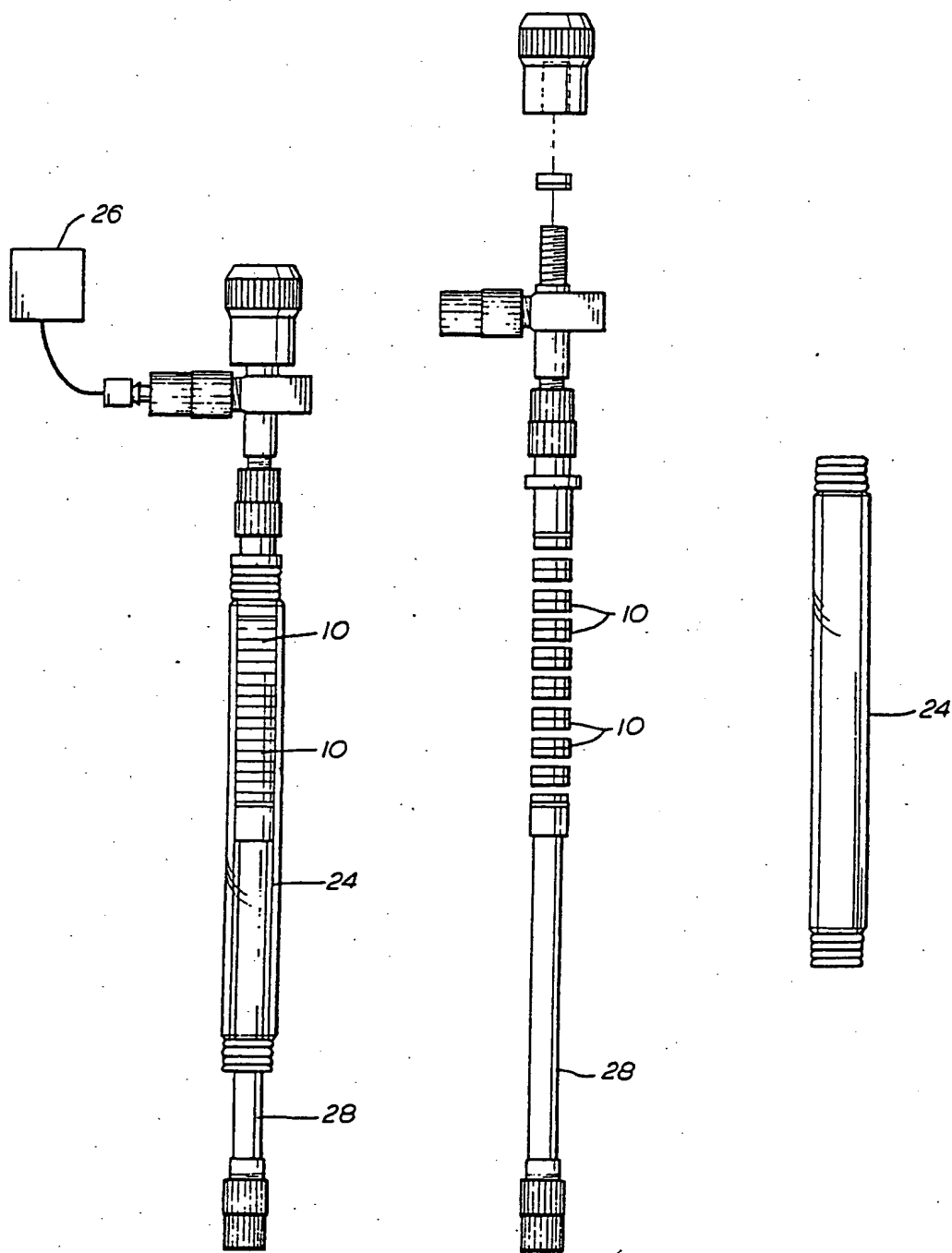


FIG. 4

SUBSTITUTE SHEET

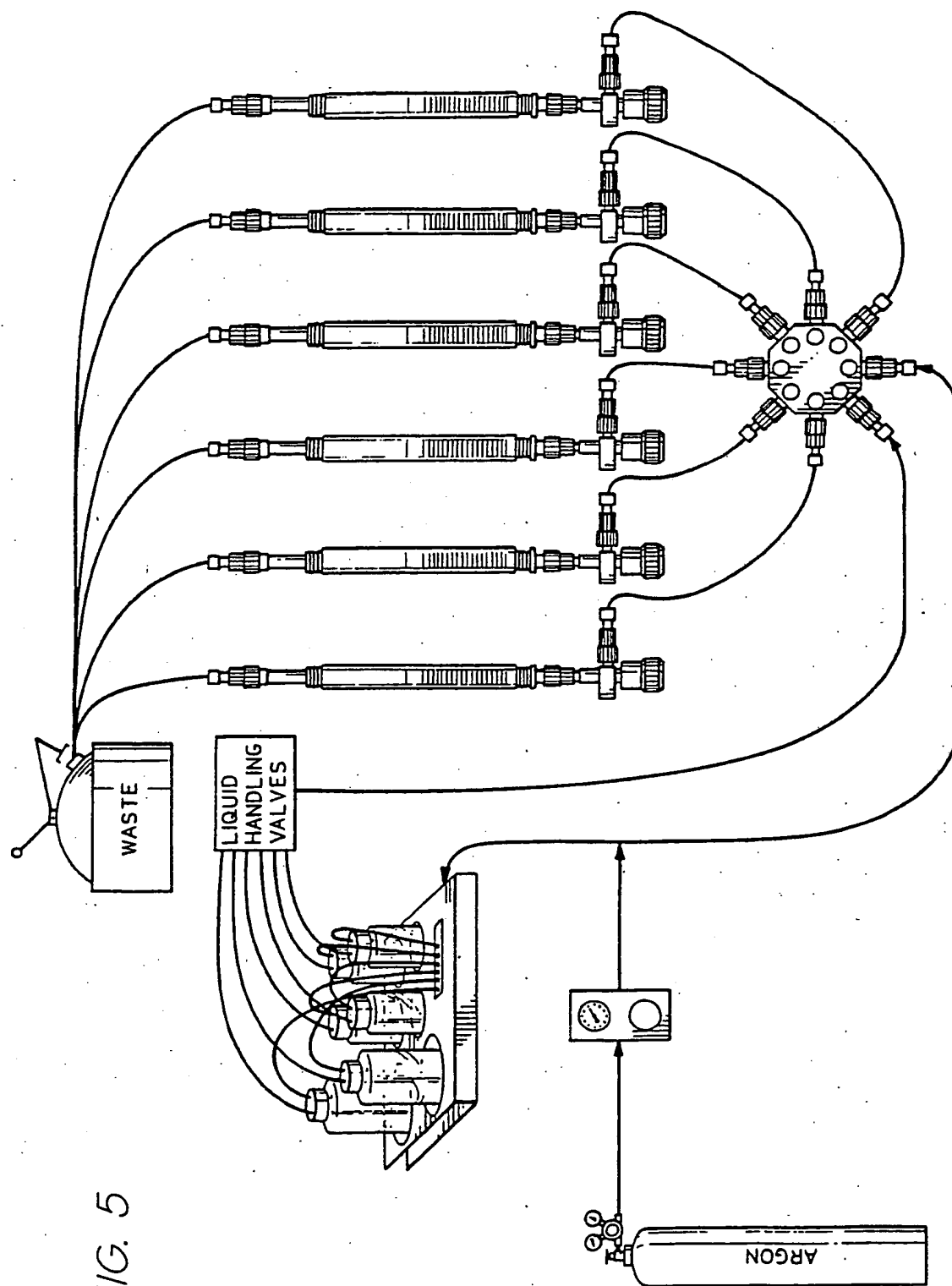


FIG. 5

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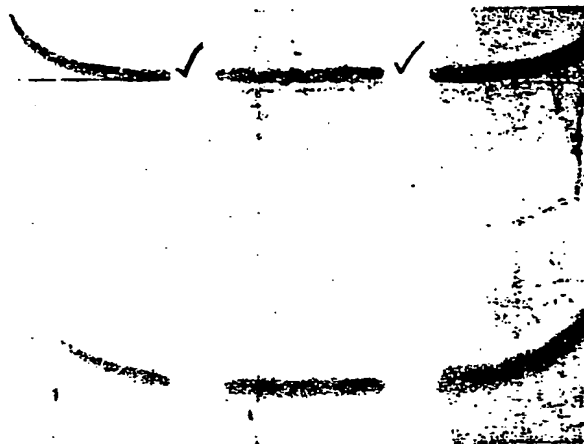


FIG. 6

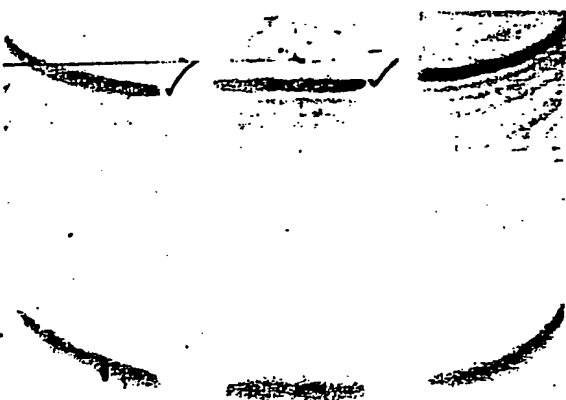


FIG. 7

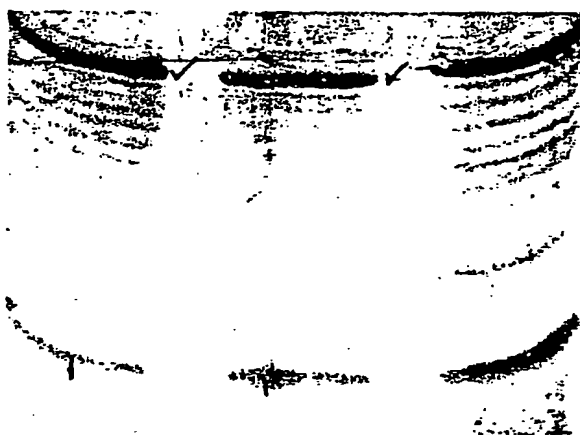


FIG. 8

I. CLASSIFICATION OF SUBJECT MATTER (In several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC(4): C12M 1/00, B01J 8/02		
U.S.CL: 422/69,116,131; 436/89; 210/198.2		
II. FIELDS SEARCHED		
Minimum Documentation Searched *		
Classification System	Classification Symbols	
U.S.	210/198.2, 501.2; 422/69, 70, 81, 116, 131, 171, 191, 241 436/89	
Documentation Searched other than Minimum Documentation to the extent that such documents are included in the fields searched *		
III. DOCUMENTS CONSIDERED TO BE RELEVANT *		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
X	US, A, 4,301,139 (FEINGERS) 17 November 1981 See entire document	1,2,6,7,14,71
X	US, A, 4,155,846 (NOVAK) 22 May 1979 See entire document.	4,16
X Y	US, A, 3,763,879 (JAWOREK) 09 October 1973 See entire document.	1-6,11,14 7-10,12,13,16
X	US, A, 3,647,390 (KUBODERA) 07 March 1972 See vessel 6.	1,2,6-8,10
X	EP, A, 164,206 (COLE) 12 November 1985 See entire document.	1,2,6-11,14,16
X,P	US, A, 4,678,769 (KING) 07 July 1987 See Entire document.	1-3,6,8,10,11
<p>* Special categories of cited documents: **</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
03 March 1988		13 APR 1988
International Searching Authority		Signature of Authorizing Officer
ISA/US		MICHAEL S. MARCUS

